Altered Activities of Anti-atherogenic Enzymes Lecithin:cholesterol Acyltransferase, Paraoxonase and Platelet-activating Factor Acetylhydrolase in Atherosclerosis Susceptible Mice

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Short running title: Anti-atherogenic enzymes in atherogenic mice

Abbreviations: PON1, paraoxonase 1; PAF-AH, platelet-activating factor acetylhydrolase; LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoprotein; LDL, low density lipoprotein; IDL, intermediate density lipoprotein; VLDL, very low density lipoprotein; DMPC, dimyristoylphosphatidylcholine;

LysoPC, lysophosphatidylcholine; POVPC,1-palmitoyl-2-oxovaleryl-*sn*-glycero-3-phosphocholine; PGPC, 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine

Abstract

We examined whether the putative anti-atherogenic enzymes lecithin:cholesterol acyltransferase (LCAT), paraoxonase 1 (PON) and platelet-activating factor acetylhydrolase (PAF-AH) are impaired in 8 week old atherosclerosis susceptible *Apoe-/-* and *Ldlr-/-* mice and whether plasma concentrations of bioactive oxidized phospholipids accumulate in plasma. *Apoe-/-* mice had reduced (28%) LCAT activity and elevated LysoPC and bioactive oxidized phospholipids (POVPC and PGPC) compared to controls on the chow diet. Elevated oxidized phospholipids and reduced LCAT activity may, in part, contribute to spontaneous lesions in these mice on chow diet. A Western diet decreased LCAT activity further (50% of controls) and PON activity was decreased 38%. The *Ldlr-/-* mice showed normal LCAT activity on chow diet and little accumulation of oxidized phospholipids. On a Western diet *Ldlr-/-* mice had reduced LCAT activity (21%), but no change in PON activity. All genotypes had reduced PAF-AH activity on the Western diet. *Apoe-/-* and *Ldlr-/-* mice, but not controls, had elevated plasma bioactive oxidized phospholipids on the Western diet. We conclude that impairment of LCAT activity and accumulation of oxidized phospholipids are part of an early atherogenic phenotype in these models.

Supplementary key words: apoE deficient mice, LDL receptor deficient mice, high density lipoproteins, atherogenic diet, bioactive oxidized phospholipids.

Introduction

It is well known that there is an inverse relationship between high density lipoprotein (HDL) and risk for cardiovascular disease in humans. HDL transports at least three enzymes including lecithin:cholesterol acyltransferase (LCAT), paraoxonase (PON) and platelet-activating factor acetylhydrolase (PAF-AH), that have putative protective roles in atherosclerosis.

LCAT, which is secreted by the liver, plays a critical role in reverse cholesterol transport, a process whereby excess cholesterol is removed from macrophage-foam cells and esterified on HDL and returned to the liver for catabolism. We have previously shown that LCAT is extremely sensitive to oxidation events in vitro and is rapidly inhibited by low levels of phospholipid hydroperoxides in plasma (1,2). A deficiency of LCAT can predispose to atherosclerosis since it has been reported that two novel missense mutations in LCAT were associated with reduced LCAT activity and premature coronary artery disease (3). Protection against atherosclerosis by LCAT is supported by the observation that overexpression of human LCAT in the transgenic rabbit on an atherogenic diet reduced not only plasma cholesterol levels but also lesion formation (4). However, overexpression of LCAT in the transgenic mouse was found to be associated with the accumulation of dysfunctional HDL and increased diet induced atherosclerosis (5). By introducing the CETP gene into the LCAT transgenic mouse, pro-atherogenic effects of elevated LCAT were corrected (6).

Taken together, these observations in humans and animal models suggest that LCAT has anti-atherogenic properties.

Paraoxonase 1 (PON), which is also synthesized and secreted by the liver (7), is believed to inactivate phospholipid hydroperoxides formed during early events of lipoprotein oxidation (8-10). Low plasma PON activity has been demonstrated in C57BL/6 mice maintained on an atherogenic high fat, high cholesterol, cholic acid diet (11); moreover, PON deficient C57BL/6 mice have increased aortic lesions compared to wild type (12). The deficiency in PON was also associated with an increase in HDL lipid hydroperoxides. Studies on PON /apoE double knockout mice suggested that PON deficiency promotes LDL oxidation and atherosclerosis; bioactive oxidized phospholipids were increased in the lipoproteins of these mice (13). These results, together with those from epidemiological studies in humans (see review,14), suggested that there is an inverse relationship between PON activity and atherosclerosis.

The enzyme, PAF-AH, secreted by macrophages hydrolyzes platelet-activating-factor (PAF) and other PAF-like lipids that are potent mediators of inflammation (15). PAF-AH in humans is associated primarily with LDL (16) although a small fraction (15%) of the enzyme is found in the HDL density range; however, in mice this enzyme is transported almost exclusively on HDL (17,18). Platelet activating factor and oxidatively fragmented phospholipids appear to be

substrates for the enzyme (19). Watson et al. (10) have shown that this enzyme is able to hydrolyze oxidized phospholipids associated with minimally oxidized LDL thus preventing monocyte binding to endothelial cells and production of monocyte chemotactic protein-1.

Two strains of mice have been extensively used to study atherosclerosis, the apoE deficient strain that is an inflammatory model demonstrating spontaneous atherosclerosis on a chow diet and exacerbated lesion formation on an atherogenic Western diet (20,21) and the LDL receptor deficient model that exhibits severe atherosclerosis on the Western diet, but not on chow (22). In the present study we examined these two atherosclerosis-prone mouse strains to test the premise that short-term exposure of the mice to an atherogenic Western diet will alter LCAT, PAF-AH and PON activities. Although there are reports that PON activity is reduced in these mice (11, 23, 24), there is little information on effects of pro-inflammatory conditions on LCAT and PAF-AH. The effect of pro-inflammatory/pro-atherogenic events in atherosclerosis-prone mouse models has not been previously examined in young (8 weeks) mice after only a short exposure to an atherogenic diet. Elucidation of early changes in the activities of the putative anti-atherogenic enzymes could be helpful in understanding the atherosclerosis susceptibility in these strains. It is likely that decreases in the functionality of the anti-atherogenic enzymes will be associated with the accumulation of bioactive oxidized phospholipids in the plasma.

Materials and Methods

Mice: C57BL/6 (controls), apoE deficient (*Apoe-/-*) and LDL receptor deficient (*Ldlr-/-)* mice, the latter two strains in the C57BL/6 background, were obtained from Jackson Laboratory at six to seven weeks of age. Only male animals were utilized. All mice were acclimated for one week before use.

Diet protocols: To test whether there were major differences in the response of LCAT, PON and PAF-AH activities between different genetic strains of atherosclerosis-prone mice, animals were maintained on a chow diet and then switched to a high fat Western diet (Teklad, 42% fat, 0.15% cholesterol) for two weeks. The two week feeding period was chosen to be more representative of early events that would predispose to atherosclerosis; this diet is atherogenic for both *Apoe-/-* and *Ldlr-/-* mice but not for the C57BL/6 controls since there is no cholate in the diet. Fasted blood samples were obtained before and two weeks after the onset of the diet and were used to determine plasma lipid concentrations and LCAT, PON and PAF-AH activities. After the two week Western diet period, the mice were switched back to a chow diet for two weeks (assessment of plasma lipid concentrations verified that these had returned to pre-Western diet levels) and subsequently each group of ten animals was separated into two groups of five; one group continued on the chow diet and the other was given the Western diet for two weeks. At the end of this period, blood

was obtained for determining accumulation of bioactive oxidized phospholipids. The animals were sacrificed and their livers removed and snap frozen in liquid nitrogen.

Plasma lipids: Plasma lipid concentrations were determined enzymatically as previously described (17). Agarose gel electrophoresis, using Lipogels (Beckman) essentially as described by the manufacturer, was used to determine the distribution of alpha, beta and pre-beta lipoproteins.

Enzyme activities: For PON activity, blood samples were drawn into heparinized tubes and activity was assayed with both phenylacetate (arylesterase activity) and paraoxon as substrate according to the procedure of Gan et al. (25). The data presented is based on arylesterase activity where one unit = 1 μmole phenylacetate hydrolyzed per min. For PAF-AH activity, blood samples were drawn into EDTA tubes and plasma was diluted 1:150 before carrying out the assay. PAF-AH activity was determined by the release of [3H]acetate from 2-acetyl-[3H]PAF essentially as described by McCall et al. (16). Results are expressed as nmoles acetate released per hr per ml. LCAT activity was measured using the exogenous proteoliposome substrate containing [14C] cholesterol as described by Chen and Albers (26). Samples were incubated for 0.5 hr and cholesterol and cholesteryl esters extracted and separated on silica gel plates.

Results are expressed as the amount of cholesterol converted to cholesteryl ester in 0.5 hr.

Quantification of plasma bioactive oxidized phospholipids: Animals were fasted before blood was obtained. Mouse plasmas from wild type (WT) control, *Ldlr-/-* and *Apoe-/-* mice on chow or Western diet were prepared by the immediate addition of BHT, EDTA and AEBSF (0.3 mM, 100 mM and 3 mM, respectively). Plasma samples were extracted with chloroform/methanol (2:1) and the chloroform layer collected.

Dimyristoylphosphatidylcholine (DMPC) was used as an internal standard. Extracted lipids were subjected to quantitative electrospray mass spectrometric analysis as described (27-29). Phospholipids were quantified based on their ion intensity relative to the internal DMPC standards; results are expressed as µg DMPC equivalents/100 µl plasma. The phospholipids quantified included: lysophosphatidylcholine (LysoPC), 1-palmitoyl-2-oxovaleryl-*sn*-glycero-3-phosphocholine (POVPC), and 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (PGPC). A total of 5 animals per group were analyzed.

Real time quantitative mRNA for PON, apoA-I and LCAT: Total RNA was determined for three of each WT, *Ldlr-/-* and *Apoe-/-* mice on chow and Western diet. The total RNA fraction was extracted from frozen, pulverized tissue using

RNeasy Miniprep Kits (Qiagen, CA). The concentration of RNA was obtained from A_{260} measurements and stored at –70°C. The primers were designed for products 300bp in length; LCAT: forward 5′-tatgtgatggggctgcctg-3′, reverse 5′-gctgtggttgtagacaatcctg-3′; apoA-I: forward 5′-ttggatatctcgcactttagc-3′, reverse 5′-agggaagagaacagtgggaat-3′; PON: forward 5′-tcagccactagtcctgtctcag-3′, reverse 5′-tgcctgcagctggcttgtcacag-3′. The β-actin gene, forward 5′-gtccacacccgcc accaattcgccatg-3′, reverse 5′-ggtgtaaaacggatcccagtaacagtccg-3′), was quantified and used as an internal standard .

Real time RT-PCR was performed in triplicate on total RNA samples or mRNA standards in 96-well optical plates on the iCycler, Thermal Cycler (Bio-Rad, CA); data was obtained using the iCycler iQ Real-Time Detection System Software, (version 2.1). Real-time one-step RT-PCR kits were purchased from Qiagen and used as directed. Each 50 µl reaction contained 25 µl 2 x QuantiTect SYBR Green RT-PCR Master Mix, 5 µl forward primer (0.5 µM), 5 µl reverse primer (0.5 µM), 0.5 µl QuantiTect RT Mix, 1 µl Uracil-N-glycosylase (heat-labile), 0.5-2.5 µl Template RNA (200 ng), 10-12 µl RNase-free water. RT-PCR parameters were as follows: reverse transcription at 50^0C for 30 min, PCR initial activation at 95^0C for15 min, 45 cycles at 94^0C for 15 s, 60^0C for 30 s and 72^0C for 30 s. Standard curves, in triplicate, were generated using serial dilutions (10-10^6) of known quantities of mouse liver mRNA (Clontech, CA). The number of standard RNA molecules was calculated essentially as described by Medhurst et al. (30).

Statistical analysis: Statistical analyses were performed using analysis of variance (ANOVA) with Scheffe's method for multiple comparisons between groups. All significance levels are two-sided. The paired t-test was used for within group comparisons. Results are expressed as mean±SD. Statistical significance is defined as a *P* level equal to, or less than, 0.05.

Results

Plasma lipoprotein distribution and lipid concentrations in control, *Apoe-/-* and *Ldlr-/-* mice: The electrophoretic mobility of lipoproteins from mice maintained on the chow diet is shown in Figure 1. Characteristically, *Apoe-/-* plasma shows a substantial increment in the pre-beta (VLDL/IDL) region and a decrement in alpha (HDL) migrating particles compared to control C57BL/6 mice. *Ldlr-/-* mice, on the other hand, show a pronounced increase in beta (LDL) particles and an increase in HDL. A similar pattern persists after the animals are placed on a Western diet except that the lipid stain is more intense (data not shown). Plasma lipid concentrations are shown in Table 1. On a chow diet triglyceride concentrations are significantly increased in both *Apoe-/-* and *Ldlr-/-* mice compared to controls; however, the greatest differences are in total cholesterol where there is a 4.4-fold and a 2.6-fold increase in *Apoe-/-* and *Ldlr-/-*, respectively, compared to controls. These increases are consistent with those previously reported (20-22). There is a small (15%) but not significant decrease HDL cholesterol in *Apoe-/-* mice on the chow diet compared to controls; on the other hand, HDL cholesterol concentrations are significantly increased (67%; P<.0001) in *LDLr-/-* mice. The Western diet increased total cholesterol (75%) and HDL cholesterol (53%) in the control mice compared to the chow diet. Two weeks maintenance on a Western diet, however, had a profound effect on total cholesterol in *Apoe-/-* and *Ldlr-/-* mice where TC doubled in the former and increased 3.6-fold in the latter over chow values. HDL cholesterol in *Apoe-/-* mice

shows a 50% decrease after two weeks on the Western diet while HDL cholesterol concentrations in *Ldlr-/-* mice were not affected. It is also apparent in Table 1 that triglyceride increases almost 6-fold in *Ldlr-/-* mice. The data indicate that short term feeding of the Western diet is associated with substantial changes in plasma lipids in the atherosclerosis prone mouse models and in modest changes in the control mice.

PON, PAF-AH and LCAT activities in control, *Apoe-/-* and *Ldlr-/-* mice: Figure 2 reveals that in chow fed mice, there is no difference in PON activity between controls and *Apoe-/-* mice; but *Ldlr-/-* mice have significantly increased (33%) activity compared to controls concomitant with elevated HDL cholesterol (Table 1). On the Western diet, PON activity increases 40% in control animals and appears to parallel the increase seen in HDL cholesterol (Table 1). PON activity in *Ldlr-/-* mice on the Western diet did not increase in activity mirroring the lack of change in HDL cholesterol. There was a modest (17%) but significant ($P<0.05$) decline in PON activity in Apoe-/- mice after two weeks on the Western diet. However, compared to controls on the Western diet, *Apoe-/-* mice exhibit a substantial decrease (38%) in PON activity on the atherogenic diet.

PAF-AH activity in *Apoe-/-* mice on either chow or Western diet was no different from controls; however, on each diet, *Ldlr-/-* mice had activity levels significantly exceeding that of controls (Figure 2). It is important to note, however, that PAF-

AH activity was significantly lower (35-40%, P<.01) on the Western diet compared to chow for all genotypes.

Unlike the other two anti-atherogenic enzymes, LCAT is significantly lower (28%, P<.001) in chow fed *Apoe*-/- mice than in controls; on a Western diet there is a 50% loss of activity (Figure 2). In contrast, the *Ldlr*-/- mice on a chow diet show no change in LCAT activity compared to controls but have a significant decrease (21%, P<.0001) in LCAT activity on the Western diet. Taken together, the data suggests that LCAT activity may be more sensitive to potential oxidative stress than either PON or PAF-AH.

Plasma accumulation of bioactive phospholipids: Because plasma lipid levels are markedly elevated in the atherosclerosis susceptible mice on a Western diet, we asked the question whether elevation of lipids is associated with an accumulation of plasma bioactive oxidized phospholipids. Figure 3 shows the concentrations of the bioactive oxidized phospholipids in mouse plasma from animals on a chow or Western diet. The data suggest that the *Apoe*-/- mice have a particularly large burden of plasma oxidized phospholipids even on a chow diet since POVPC and PGPC are substantially elevated (288% and 80%, respectively) over controls. In contrast, *Ldlr*-/- mice do not have elevated oxidized phospholipids on the chow diet. Both genotypes exhibit a 35% increase in plasma LysoPC concentrations compared to controls.

As shown in Figure 3, consumption of the Western diet is associated with significant increases in plasma bioactive oxidized phospholipids in all genotypes. *Apoe-/-* mice on the Western diet, however, have significantly higher concentrations of LysoPC, POVPC, and PGPC than controls on the Western diet while *Ldlr -/-* mice have greater quantities of LysoPC and POVPC than controls. The data demonstrate that the Western diet is associated with increased levels of plasma oxidized phospholipids and that *Apoe-/-* and *Ldlr-/-* mice have especially elevated levels of these bioactive phospholipids.

mRNA levels of PON, apoA-I and LCAT: LCAT is expressed primarily in the liver which is also the major site of apoA-I and PON synthesis. Since *Apoe-/-* mice have a significant loss of LCAT and PON activity, as well as low HDL cholesterol concentrations on the Western diet, we examined, by real time quantitative PCR, whether mRNA levels for LCAT, PON and apoA-I were altered in these mice compared to the other genotypes and whether diet had an impact on mRNA levels. The expression levels of apoA-I, LCAT and PON in the liver of each genotype is shown as copies/ng total mRNA in Figure 4. The data reveal that there are no differences in LCAT, PON and apoA-I mRNA expression between the different genotypes nor does the Western diet influence mRNA levels. The absence of any significant reduction in mRNA levels for LCAT and

PON in *Apoe-/-* mice suggests that post-transcriptional events are responsible for low enzyme activities.

Diet-related changes in the distribution of PAF-AH activity: Unlike humans where PAF-AH is associated primarily with LDL, PAF-AH in the mouse is associated almost exclusively with HDL particles. Earlier studies with LCAT deficient mice suggested to us that PAF-AH can associate with LDL-like particles under metabolic conditions where LDL is elevated (17). To test whether the change in lipoprotein distribution changes the distribution of PAF-AH activity, pooled plasma from control, *Apoe-/-* and *Ldlr-/-* mice on chow and Western diet were subjected to fast protein liquid chromatography and enzyme activity of the fractions determined. Figure 5 summarizes the data and reveals that on a Western diet the *Ldlr-/-* mice have a modest amount (16%) of PAF-AH activity associated with LDL (fractions 11-15) while only scant or no activity is in this fraction in *Apoe-/-* and control mice, respectively. The cholesterol concentration in *Ldlr-/-* mice increased 3.7-fold on the Western compared to chow diet suggesting that large increases in LDL cholesterol levels may coincide with the shift of PAF-AH into this density region.

Discussion

The enzymes, PON and PAF-AH, are reputed to have anti-oxidative properties and by virtue of this property are thought to be athero-protective. PON has been shown to hydrolyze oxidized phospholipids that are pro-inflammatory agents (10) and to protect LDL from oxidation (8) thus preventing monocyte recruitment and cell adhesion (23). In human subjects, low PON activity has been associated with increased risk for coronary artery disease (31-34) while a deficiency of PON in C57BL/6 mice is coupled with increased lesion formation in mice maintained on an atherogenic diet (12). PAF-AH is also known to hydrolyze pro-inflammatory oxidized phospholipids (19,35). Several recent studies have shown that PAF-AH is elevated in subjects who had coronary events suggesting that PAF-AH is a marker for atherosclerosis and could potentially be a contributing factor to coronary artery disease (36,37). However, it is unclear whether elevated PAF-AH activity is a contributing factor in atherosclerosis since studies with Japanese subjects who are deficient in PAF-AH indicate that CAD is increased in this population (38). A more direct approach with *Apoe-/-* mice subjected to adenovirus-mediated gene transfer of human PAF-AH indicated that over-expression of the enzyme lowered ICAM-1 and VCAM-1 expression and diminished βVLDL-induced macrophage adhesion suggesting an athero-protective role for PAF-AH in this model (39).

Although it has been suggested that LCAT, by virtue of its phospholipase activity, has anti-oxidative properties (40,41), its primary function in athero-protection is thought to be through its participation in reverse cholesterol transport (42). In the present study we examined whether these three anti-atherogenic enzymes are impaired in atherosclerosis susceptible mice placed on an atherogenic diet for a short duration and whether changes in enzyme function are associated with an increase in plasma concentrations of pro-inflammatory bioactive oxidized phospholipids.

Our studies with *Apoe-/- mice* reveal that even on a chow diet, there is a significant elevation of LysoPC and the bioactive phospholipids, POVPC and PGPC; such an accumulation may contribute to spontaneous lesion formation in these mice on a chow diet. Interestingly, LCAT activity was also significantly decreased; this loss of activity could, in part, be caused by the accumulation of oxidized lipids. We have previously shown that plasma LCAT activity *in vitro* is extremely sensitive to, and readily inhibited by, the accumulation of low levels of oxidation products (1,2). Reduction in LCAT activity would be expected to exacerbate the atherosclerotic process since cholesterol efflux from macrophage/foam cells would likely be impaired.

The phospholipid composition of lipoproteins in the *Apoe-/-* mouse is unusual because sphingomyelin is highly enriched in all lipoprotein fractions (43). It has

also been reported that sphingomyelin is an inhibitor of LCAT activity (44); therefore, an alternative explanation for the loss of LCAT activity in *Apoe-/-* mice on the chow diet is that some of this loss may be attributable to the increased sphingomyelin to phosphodidylcholine ratio. Although the major function of LCAT is the esterification of cholesterol, it is possible that the reduction of LCAT activity in these mice, as well as in *Ldlr-/-* mice on the Western diet, may contribute to the accumulation of oxidized phospholipids since Goyal et al. (41) showed that LCAT has the capacity, albeit weak, to hydrolyze oxidized phospholipid, *in vitro*.

On the Western diet there were significant reductions in PON, PAF-AH and LCAT activities in *Apoe-/-* mice which were paralleled by large increases in the bioactive oxidized phospholipids. We examined mRNA levels for LCAT, apoA-I and PON, all of which are synthesized by the liver, but found no change related to the atherogenic diet suggesting changes in enzyme activity are due to post translational events. HDL cholesterol concentration in these mice decreased 50% on the Western diet; since PON, PAF-AH and LCAT enzymes are transported on HDL this reduction could conceivably decrease enzyme activity. On the other hand, HDL was elevated in control mice on the Western diet and concomitantly LCAT activity was not inhibited suggesting that increased HDL levels may protect enzyme function. It is interesting that Zhang et al. (20) reported a decrease in HDL in *Apoe-/-* mice on an atherogenic diet but found that apoA-I

concentrations were not affected but rather that apoA-I distribution was altered. They found that a large proportion of apoA-I was associated with less dense lipoproteins rather than HDL. We too noted a similar phenomenon where, on a Western diet, apoA-I was associated with IDL/LDL and VLDL in *Apoe-/-* mice (data not shown). It has been suggested that apoA-I may be required for optimal PON activity since this protein co-isolates with PON1 during purification of the enzyme (45). We have also demonstrated that the conformation of apoA-I appears to play a role in optimizing PON activity (46). Therefore, it is conceivable that an altered apoA-I conformation that can be expected by its transport on non-HDL particles may adversely affect PON activity in *Apoe-/-* mice. Alternatively, PON activity may be reduced because the enzyme is impaired by the accumulation of oxidized phospholipids; indeed, inhibition of HDL-PON activity by copper oxidation was demonstrated *in vitro* by Aviram et al. (47). The redistribution of apoA-I can, as well, potentially contribute to the decreased LCAT activity since apoA-I is a co-factor in activation of LCAT. Although speculative, it is possible that the concomitant decline in the activity of the anti-atherogenic enzymes together with an increase in bioactive oxidized phospholipids contribute to the pronounced lesions found in *Apoe-/-* mice on an atherogenic diet. This speculation is supported by the observations of Watson et al. (27) that POVPC and PGPC are found in the atherosclerotic lesions of cholesterol fed rabbits and that both these bioactive oxidized phospholipids induce endothelial cells to bind monocytes *in vitro*.

Unlike *Apoe-/-* mice that develop spontaneous lesions on a chow diet, the *Ldlr-/-* mouse does not develop atherosclerosis under this condition. This may be explained, in part, by the observations: (1) that the total plasma burden of bioactive oxidized lipids is less in the *Ldlr-/-* genotype than in the *Apoe-/-* genotype and (2) that there is a significant increase in PON and PAF-AH activities compared to WT which parallels the increase in HDL cholesterol in the *Ldlr-/-* mouse. Although speculative, the latter may be important in protecting the anti-atherogenic function of HDL such as LCAT activity which, unlike that of *Apoe-/-* mice, is not reduced in chow fed *Ldlr-/-* mice.

In the *Ldlr-/-* mouse, consumption of the Western diet resulted in a markedly increased accumulation of POVPC compared to controls. Although oxidized phospholipids increased significantly, PON activity, unlike that seen in *Apoe-/-* mice, was unaffected. In control mice on the Western diet there was actually an increase in PON activity; this increase mirrored an increase in HDL cholesterol suggesting that increased HDL concentrations may preserve PON function.

Ldlr-/- mice on the Western diet were unusual because PAF-AH activity was significantly elevated over controls even though both genotypes had similarly elevated HDL. Clearly, elevated HDL levels are not sufficient in themselves to maintain PAF-AH activity. It was previously demonstrated by Stafforini et al.

(48) that, in humans, the lipoprotein environment in which PAF-AH exists influences its catalytic activity. They noted that, in humans, activity was most efficient on LDL particles. A possible explanation for the elevation of PAF-AH activity in *Ldlr-/-* mice on the Western diet compared to that of controls is that redistribution of some of the enzyme's activity (16%) to LDL may be important in maintaining the stability and activity of the enzyme during fat feeding.

The reductions of PAF-AH and PON activities in *Apoe-/-* and PAF-AH activity in *Ldlr-/-* mice on the Western diet may, in part, be due to inactivation of these enzymes by the oxidized phospholipids in the plasma. This is supported by *in vitro* studies that have shown that oxygen free radicals generated by xanthine/xanthine oxidase (49) and copper-mediated oxidation of LDL (50) inhibit plasma PAF-AH and that copper ion-induced oxidation products inhibit PON1 (47). In the present study, however, *Apoe-/-* and *Ldlr-/-* mice on the chow diet have a modest increase in bioactive oxidized phospholipids that is not associated with decreased PON1 and PAF-AH activity. It is likely that in the closed *in vitro* systems the response is more pronounced and that *in vivo* the threshold for oxidative inhibition of the enzymes is increased.

The results from the two different atherosclerosis susceptible mouse strains suggest that the response to an atherogenic diet is complex. However, it is likely that the total plasma burden of bioactive oxidized phospholipids is important in

modulating the pro-inflammatory responses that predispose to atherosclerosis. Thus, it is not surprising that *Apoe-/-* mice that have precocious atherosclerosis on the chow diet also have a greater burden of bioactive oxidized phospholipids than *Ldlr-/-* mice that do not develop spontaneous atherosclerosis on chow. This study also suggests that the anti-atherogenic enzyme, LCAT, is more sensitive to plasma oxidative events than either PON and PAF-AH since it was the only enzyme that was significantly decreased in both atherosclerosis susceptible mouse models compared to controls. It is tempting to speculate that impairment of LCAT, and subsequently cholesterol efflux, by the accumulation of plasma bioactive oxidized phospholipids may be a key event in development of atherosclerotic lesions in these models.

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REFERENCES

1. Bielicki, J.K., T.M. Forte and M.R. McCall, 1996. Minimally oxidized LDL is a potent inhibitor of lecithin: cholesterol acyltransferase activity. *J. Lipid Res.* **37**: 1012-1021.

2. Bielicki, J.K. and T.M. Forte, 1999. Phospholipid hydroperoxides inhibit plasma lecithin: cholesterol acyltransferase activity. *J. Lipid Res*. **40**: 1-7.

3. Kuivenhoven, J.A., A.F.H. Stalenhoef, J.S. Hill, P.N.M. Demacker, A. Errami, J.J.P. Kastelein, and P.H. Pritchard. 1996. Two novel molecular defects in the *LCAT* gene are associated with Fish Eye Disease. *Arterioscler. Thromb. Vasc. Biol*. **16**: 294-303.

4. Hoeg, J.M., S. Santamarina-Fojo, A.M. Berard, J. F. Cornhill, E.E. Herderick, S.H. Feldman, C.C. Haudenschild, B.L. Vaisman, R.F. Hoyt, Jr, S.J. Demosky, Jr., R. D. Kauffman, C.M. Hazel, S.M. Marcovina and H.B. Brewer, Jr. 1996. Overexpression of lecithin:cholesterol acyltransferase in transgenic rabbits prevents diet-induced athersoclerosis. *Pro. Natl. Acad. Sci.* USA **93**: 11448-11453.

5. Berard, A.M., B. Foger, A.Remaley, R. Shamburek, B.L. Vaisman, G. Talley, B. Paigen, R.F. Hoyt, S. Marcovina, H.B. Brewer and S. Santamarina-Fojo. 1997. High plasma HDL concentrations associated with enhanced atherosclerosis in transgenic mice overexpressing lecithin:cholesterl acyltransferase. *Nat. Med*. **3**: 744-749.

6. Foger, B., M. Chase, M.J. Amar, B.L. Vaisman, R.D. Shamburek, B. Paigen, J.Fruchart-Najib, J.A. Paiz, C.A. Koch, R.F. Hoyt, H.B.Brewer,

Jr., and S. Santamarina-Fojo. 1999. Cholesteryl ester transfer protein corrects dysfunctional high density lipoproteins and reduces aortic atherosclerosis in lecithin cholesterol acyltransferase trnasgenic mice. *J. Biol. Chem*. **274**: 36912-36920.

7. LaDu, B.N., 1992. Human serum paraoxonase/arylesterase. *In* Pharmacogenetics of Drug Metabolism, Pergamon Press, Inc., New York, N.Y. 51-91.

8. Mackness, M.I., S. Arrol, C. Abbott and P.N. Durrington, 1993. Protection of low-density lipoprotein against oxidative modification of high-density lipoprotein associated paraoxonase. *Atherosclerosis*. **104**: 129-135.

9. Mackness, M. I. and P.N. Durrington, 1995. HDL, its enzymes and its potential to influence lipid peroxidation. *Atherosclerosis.* **115**: 243-253.

10. Watson, A.D.; J.A. Berliner, S.Y. Hama, B.N. LaDu, K.F. Faull, A.M. Fogelman and M. Navab. 1995. Protective effect of high density lipoprotein associated paraoxonase. Inhibition of the biological activity of minimally oxidized low density lipoprotein. *J. Clin. Invest*. **96**: 2882-2891.

11. Shih, D.M.; L. Gu, S. Hama, Y.R. Xia, M. Navab, A.M. Fogelman and A.J. Lusis. 1996. Genetic-dietary regulation of serum paraoxonase expression and its role in atherogenesis in a mouse model. *J. Clin. Invest*. **97**: 1630-1639.

12. Shih, D.M.; L. Gu, Y.R. Xia, M. Navab, W.F. Li, S. Hama, L.W. Castellani, C.E. Furlong, L.G. Costa, A.M Fogelman and A.J. Lusis. 1998. Mice lacking serum paraoxonase are susceptible to organophosphatetoxicity and atherosclerosis. *Nature*. **394**: 284-287.

13. Shih, D.M.; Y.R. Xia, X.P. Wang, E. Miller, L.W. Castellani, G. Subbanagounder, H. Cheroutre, K.F. Faull, J.A. Berliner, J.L. Witztum and A.J. Lusis. 2000. Combined serum paraoxonase knockout/apolipoprotein E knockout mice exhibit increased lipoprotein oxidation and atherosclerosis. *J. Biol. Chem*. **275**: 17527-35.

14. Durrington, P.N.; B. Mackness and M.I. Mackness. 2001. Paraoxonase and atherosclerosis. *Arterio. Throm Vasc. Biol*. **21**: 473-480.

15. Stafforini, D.M.; T.M. McIntyre, G.A. Zimmerman and S.M. Prescott. 1997. Platelet-activating factor acetylhydrolases. *J. Biol. Chem*. **272**: 17895-17898.

16. McCall, M.R., M. LaBelle, T.M. Forte, R.M. Krauss, Y. Takanami and D.L. Tribble. 1999. Dissociable and nondissociable forms of platelet-activating factor acetylhydrolase in human plasma LDL: Influence on LDL oxidative susceptibility. *Biochem. Biophys. Acta.* **1437**: 23-36.

17. Forte, T.M., M.N. Oda, L. Knoff, B. Frei, J. A.K. Harmony, W.D. Stuart, E.M. Rubin and D.S. Ng. 1999. Targeted disruption of the murine LCAT gene is

associated with reductions in plasma paraoxonase and platelet-activating factor acetylhydrolase activities but not in apolipoprotein J concentration. *J. Lipid Res*. **40**: 1276-1283.

18. Tsaoussis, V., C. Vakirtzi-Lemonias. 1994. The mouse plasma PAF acetylhydrolase: II. It consists of two enzymes both associated with the HDL. *J. Lipid Mediators and Cell Signaling*. **9**: 317-331.

19. Stremler, K.E.; D.M. Stafforini, S.M. Prescott and T.M. McIntyre. 1991. Human plasma platelet-activating factor acetylhydrolase. Oxidatively fragmented phospholipids as substrates. *J. Biol. Chem*. **266**: 11095-11103.

20. Zhang, S.H., R.L. Reddick, J.A. Piedrahita and N. Maeda. 1992. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science.* **258**: 468-471.

21. Plump, A.S., J.D. Smith, T. Hayek, K. Aalto-Setala, A.Walsh, J.G. Verstuyft, E.M. Rubin and J.L. Breslow. 1992. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell*. **71**: 343-353.

22. Ishibashi, S., M.S. Brown, J.L. Goldstein, R.D. Gerard, R.E. Hammer and J. Herz. 1994. Hypercholesterolemia and low density lipoprotein receptor

knockout mice and its reversal by adenovirus-mediated gene delivery. *J. Clin. Invest*. **92**: 883-893.

23. Navab, M.; S. Hama-Levy, B.J. Van Lenten, G.C. Fonarow, C.J. Cardinez, L.W. Castellani, M.L. Brennan, A.J. Lusis, A.M. Fogelman and B.N. LaDu. 1997. Mildly oxidized LDL induces an increased apolipoprotein J/paraoxonase. *J. Clin. Invest.* **99**: 2005-2019.

24. Aviram, M; M. Rosenblat, C.L. Bisgaier, R.S. Newton, S.L. Primo-Parmo, B.N. LaDu. 1998. Paraoxonase inhibits high-density lipoprotein oxidation and preserves its functions. A possible peroxidative role for paraoxonase. *J. Clin. Invest*. **101**: 1581-1590.

25. Gan, K. N., A. Smolen, H.W. Eckerson and B.N. LaDu. Purification of Human Serum Paraoxonase/Arylesterase. *Drug Metabol. Disp.* **19:** 100-106.

26. Chen, C. and J.J. Albers. 1982. Characterization of proteoliposomes containing apoprotein A-I: a new substrate for the measurement of lecithin: cholesterol acyltransferase activity. *J. Lipid Res*. **23**: 680-691.

27. Watson, A.D.; N. Leitinger, M. Navab, K.F. Faull, S. Hörkkö, J.L. Witztum, W. Palinski, D. Schwenke, R.G. Salomon, W. Sha, G. Subbanagounder, A.M. Fogelman and J.A. Berliner. 1997. Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density

lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo. *J. Biol. Chem*., **272**: 13597-13607.

28. Watson, A.D.; G. Subbanagounder, D.S. Welsbie, K.F. Faull, M. Navab, M.E. Jung, A.M. Fogelman, and J.A. Berliner. 1999. Structural identification of a novel pro-inflammatory epoxyisoprostane phospholipid in mildly oxidized low density lipoprotein. *J. Biol. Chem.* **274**: 24787-24798.

29. Subbanagounder, G; N. Leitinger, D.C. Schwenke, J.W. Wong, H. Lee, C. Rizza, A.D. Watson, K.F. Faull, A.M. Fogelman, J.A. Berliner. 2000. Determinants of bioactivity of oxidized phospholipids. Specific oxidized fatty acyl groups at the sn-position. *Arterio. Thromb. Vasc. Biol*. **20**: 2248-2254.

30. Medhurst, A.D., D. C. Harrison, S.J. read, C.A. Campbell, M.J. Robbins and M.N. Pangalos. 200. The use of TaqMan RT-PCR assays for semiquantitative analysis of gene expression in CNS tissues and disease models. *J. Neurosciences Meth*. **98**: 9-20

31. Abbott, C.A.; M.I. Mackness, S. Kumar, A.J. Boulton and P.N. Durrington. 1995. Serum paraoxonase activity, concentration, and phenotype distribution in diabetes mellitus and its relationship to serum lipids and lipoproteins. *Arterio. Throm. Vasc. Biol.* **11**: 1812-1818.

32. Mackness, M.I.; D. Harty, D. Bhatnagar, P.H. Winocour, S. Arrol, M. Ishola, and P.N. Durrington. 1991. Serum paraoxonase activity in familial

hypercholesterolaemia and insulin-dependent diabetes mellitus. *Atherosclerosis*. **86:** 93-199.

33. McElveen, J., M.I. Mackness, C. Colley, T. Peard, S. Warner and C.H. Walker. 1986. Distribution of Paraoxon Hydrolytic Activity in the Serum of Patients after Myocardial Infarction. *Clin. Chem*. **32**: 671-673.

34. Hedrick, C.C., S.R., Thorpe, M.X. Fu, C.M. Harper, J. Yoo, S.M. Kim, H. Wong and A.L. Peters. 2000. Glycation impairs high-density lipoprotein function. *Diabetologia.* **43**: 312-320.

35. Watson, A.D.; M. Navab, S.Y. Hama, A. Sevanian, S.M. Prescott, D.M. Stafforini, T.M. McIntyre, B.N. LaDu, A.M. Fogelman and J.A. Berliner. 1995. Effect of platelet activating factor-acetylhydrolase on the formation and action of minimally oxidized low density lipoprotein. *J. Clin. Invest*. **95**: 774-782.

36. Caslake, M.J.; C.J. Packard, K.E. Suckling, S.D. Holmes, P. Chamberlain and C.H. Macphee. 2000. Lipoprotein-associated phospholipase A(2), platelet-activating factor acetylhydrolase: a potential new risk factor for coronary artery disease. *Athero.* **150**: 413-419.

37. Packard, C.J., D.S. O'Reilly, M.J. Caslake, A.D. McMahon, I. Ford, J. Cooney, C.H. Macphee, K.E. Suckling, M. Krishna, F.E. Wilkinson, A. Rumley and G.D. Lowe. 2000. Lipoprotein-associated phospholipase A2 as an

independent predictor of coronary heart disease. West of Scotland Coronary Prevention Study Group. *N. Eng. J. Med*. **343**: 1148-1155.

38. Unno, N.; T. Nakamura, H. Kaneko, T. Uchiyama, N. Yamamoto, J. Sugatani, M. Miwa and S. Nakamura. 2000. Plasma platelet-activating factor acetylhydrolase deficiency is associated with atherosclerotic occlusive disease in Japan. *J. Vasc. Surg*. **32**: 263-267.

39. Theilmeier, G., B. deGeest, P.P.Van Veldhoven, D. Stengel, C.Michiels, M. Lox, M. Landeloos, M.J. Chapman, E. Ninio, D. Collen, B. Himpens and P.Holvoet. 2000. HDL-associated PAF-AH reduces endothelial adhesiveness in apoE-/- mice. *FASEB J*. **14**: 2032-2039.

40. Liu, M. and P.V. Subbaiah. 1994. Hydrolysis and transesterification of platelet-activating factor by lecithin-cholesterol acyltransferase. *Proc. Nat. Acad. Sci.* **91**: 6035-6039.

41. Goyal, J., K. Wang, M. Liu and P.V. Subbaiah. 1997. Novel function of lecithin:cholesterol acyltransferase: hydrolysis of oxidized polar phospholipids generated during lipoprotein oxidation. *J. Biol. Chem.* **272**: 16231-16231.

42. Glomset, M.A., G. Assmann, E. Gjone, and K.R. Norum. 1995. Lecithin:cholesterol acyltransferase deficiency and fish-eye disease. *In*, The

Metablolic Basis of Inherited Diseases. C.R. Scriver, A.L. Beaudent, W.S. Sly and D. Valle, editors. McGraw-Hill, New York, NY. 19933-1952.

43. Jeong, T., S.L. Schissel, I.Tabas, H.J. Pownall, A.R. Tall and X. Jiang. 1998. Increased sphingomyelin content of plasma lipoproteins in apolipoprotein E knockout mice reflects combined production and catabolic defects and enhances reactivity with mammalian sphingomyelinase. *J. Clin. Invest*. **101**: 905-912.

44. Subbaiah, P.V. and M. Liu. 1993. Role of sphingomyelin in the regulation of cholesterol esterification in the plasma lipoproteins: inhibition of lecithin-cholesterol acyltransferase reaction. J. Biol. Chem. **268**: 20156-20163.

45. Sorenson, R.C.; C.L. Bisgaier, M. Aviram, C. Hsu, S. Billecke and B.N. LaDu. 1999. Human serum Paraoxonase/Arylesterase's retained hydrophobic N-terminal leader sequence associates with HDLs by binding phospholipids : apolipoprotein A-I stabilizes activity. *Arterio. Throm. Vasc. Biol*. **19**: 2214-2225.

46. Oda, M.N, J.K. Bielicki, T. Berger and T.M. Forte. 2001. Cysteine Substitutions in Apolipoprotein A-I Primary Structure Modulate Paraoxonase Activity. *Biochem.* **40**: 1710-1718.

47. Avriam, M., S. Billecke, R. Sorenson, C. Bisgaier, R. Newton, M. Rosenblat, J. Erogul, C. Hsu, C. Dunlop and B. La Du. 1998. Paraoxonase active site required for protection against LDL oxidation involves its free sulfhydryl group and is different from that required for its arylesterase/paraoxonase activities: selective action of

human paraoxonase allozymes Q and R. *Arterioscler. Thromb. Vasc. Biol.* **18:** 1617-1624.

48. Stafforini, D. M., M.R. Elstad, T.M. McIntyre, G.A. Zimmerman and S.M. Prescott. 1990. Human macrophages secrete platelet-activating factor acetylhydrolase. *J. Biol Chem.,* **265**: 9682-9687.

49. Ambrosio, G., A. Oriente, C. Napoli, G. Palumbo, P. Chiariello, G. Marone, M. Condorelli, M. Chiariello and M. Triggiani. 1994. Oxygen radicals inhibit human plasma acetylhydrolase, the enzyme that catabolizes platelet-activating factor. *J. Clin. Invest*. **93**: 2408-2416.

50. Dentan, C., P. Lesnik, M.J. Chapman and E. Ninio. 1994. PAF-acether-degrading acetylhydrolase in plasma LDL is inactivated by copper- and cell-mediated oxidation. *Arterioscler. Thromb*. **14**: 353-360.

Figure Legends

Figure 1. Agarose gel electrophoresis profiles of plasma from control, *Apoe-/-* and *Ldlr-/-* mice. The figure shows the lipid profile from five mice of each genotype on the chow diet.

Figure 2. Changes in PON, PAF-AH and LCAT activities as a function of genotype and diet. Data represent the mean ± SD of ten animals. ANOVA: * $P<0.01$ and ** $P<0.0001$ compared to WT controls within each diet group.

Figure 3. Accumulation of bioactive oxidized phospholipids and lysolecithin in the plasma of control (WT), *Apoe-/-* and *Ldlr-/-* mice maintained on chow or Western diet. Values represent the mean ± SD of five animals. ANOVA: † $P<0.05$, * $P \leq 0.01$, ** $P \leq 0.001$ and *** $P \leq 0.0001$ compared with WT within each diet group. The data also reveal that oxidized phospholipids are generally significantly higher on the Western diet than on chow where paired t-test *P* values are: controls; 0.05, <0.03, NS: *Apoe-/-*; 0.04, <0.0001, 0.0002: *Ldlr-/-*; 0.0003. <0.02, 0.0001 for POVPC, PGPC and LysoPC, respectively.

Figure 4. ApoA-I, LCAT and PON mRNA expression in mouse liver. The sensitivity of RT-PCR using different primer sets was established from the cycle threshold (Ct) values obtained using known quantities of mRNA. For the

primers sets, a Ct of 22-25 was obtained with 22,000 copies. Copies/ng mRNA were calculated from standard curves constructed from serial dilutions of known quantities of mouse liver mRNA. Reverse transcription reactions from each RNA sample were carried out in triplicate. The values represent the mean ± SD from 3 mouse livers per group.

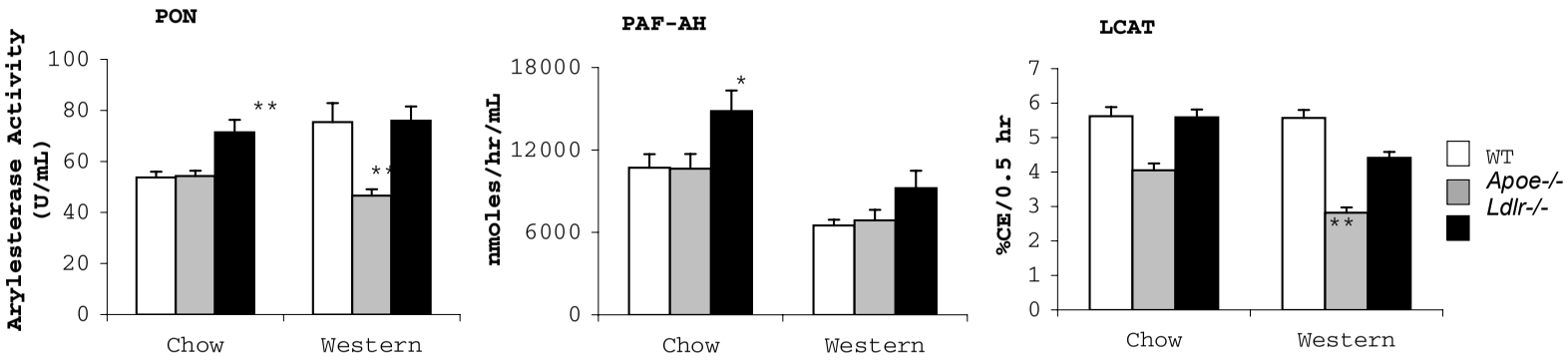
Figure 5. Distribution PAF-AH activity and plasma cholesterol in control, *Apoe-/-* and *Ldlr-/-* mice on a chow or Western diet. Pooled plasmas (1.0 ml) were fractionated by FPLC; 0.5ml fractions were collected and assayed for cholesterol and PAF-AH activity. Comparison of the cholesterol and PAF-AH activity profiles indicate that most of the enzyme activity localizes to the HDL fraction except in the case of *Ldlr-/-* mice on the Western diet. Calibration with isolated lipoproteins indicate that lipoproteins isolate as follows: VLDL, fr 5-6; IDL, fr 7-10; LDL, fr 11-15; HDL, fr 16-25. Note the change of scale for cholesterol concentrations in the Western diet profiles.

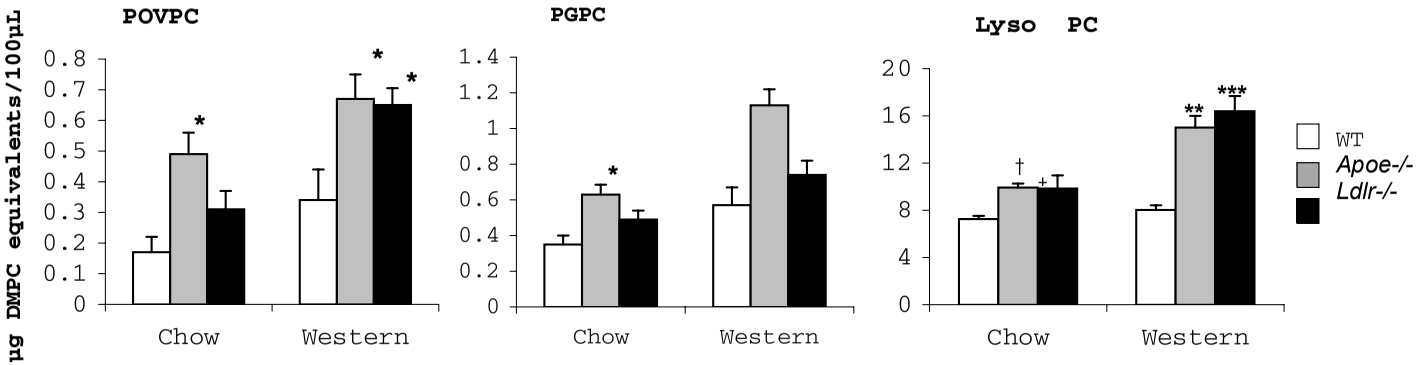
Table 1. Plasma lipid concentrations (mg/ml) in WT, *Apoe-/-*, and *Ldlr-/-* mice on chow and western diet

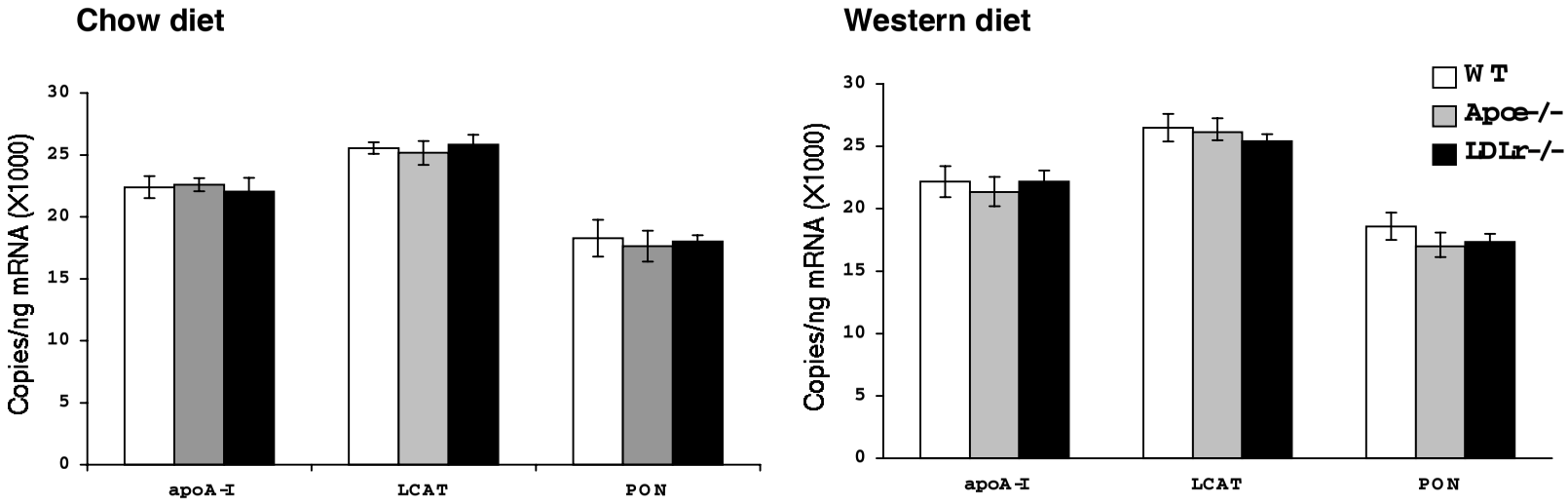
Genotype	Diet	TG	TC	HDL-C
WT	Chow	0.29±0.06	1.13±0.07	1.08±0.07
Apoe-/-	Chow	0.80±0.31**	5.01±1.01***	0.92±0.16
Ldlr-/-	Chow	0.74±0.28**	2.92+0.47***	1.80+0.23***
WT	Western	0.26±0.08	1.98±0.30	1.66±0.25
Apoe-/-	Western	0.49±0.25	11.46±2.56***	0.47±0.24*
Ldlr-/-	Western	4.22±2.42***	10.50±3.78***	1.97±0.40

Data represent the mean ±SD of 10 mice per group

Compared to WT, ANOVA: *P= 0.003; **P<0.001; ***P<0.0001


PON
Arylesterase Activity (U/mL)
100
80
60
40
20
0
**
**
Chow
Western
PAF-AH
nmoles/hr/mL
18000
12000
6000
0
*
Chow
Western
LCAT
%CE/0.5 hr
7
6
5
4
3
2
1
0
**
Chow
Western
WT
Apoe-/-
Ldlr-/-


µg DMPC equivalents/100µL
POVPC
PGPC
Lyso PC
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1.4
1.2
1
0.8
0.6
0.4
0.2
0
20
16
12
8
4
0
*
*
*
*
*
†
†
**
***
Chow
Western
Chow
Western
Chow
Western
WT
Apoe-/-
Ldlr-/-


Chow diet
Western diet
Copies/ng mRNA (X1000)
30
25
20
15
10
5
0
apoA-I
LCAT
PON
WT
Apoe-/-
LDLr-/-